Can Thymidine Kinase 1 Detect Small Invisible Malignant Tumours?

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Research

Keywords: thymidine kinase 1, serum thymidine kinase 1 concentration (STK1p), early tumour detection, invisible malignant tumour, magnetic beads, automatic chemiluminescence analyser.

Posted Date: August 31st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-839400/v1

Abstract

Objectives. Early detection of malignant tumour is a prerequisite for a successful treatment.

Here we investigate if thymidine kinase 1 is more sensitive than imaging technology to discover small invisible malignant tumours.

Material and Methods. The cellular concentration of TK1 was determined by an automatic chemiluminescence analyzer of magnetic particle immune sandwich minimum. The primary and secondary antibodies linked to the magnetic beads were chicken anti-human thymidine kinase 1 IgY-polyclonal antibodies (IgY). The minimum number of cells able to detect by the automatic chemiluminescence analyzer were determined based on the cellar TK1 concentration of low and high TK1 cell lines of known cell count.

Results. The TK1 concentration of malignant cell was found to be 0.021 pg/cell. Assuming 200 pg of total protein/cell, TK1 corresponds to 0.01 % of the total protein/cell. The concentration of TK1 in human blood serum of malignant patients is in the range of 2-10 pmol/l (pM), corresponding to about 50 $x10^6$ growing cells that release TK1 into 5 litre blood. The limit visibility by imaging of a tumour is about 1 mm in diameter, corresponding to about 10^9cells of a cell diameter of 1µm.

Conclusion. TK1 is more sensitive than imaging.

Introduction

Early detection of malignant tumour is a prerequisite for successful treatments. Therefore, imaging technologies have been developed during the last decades. However, the imaging methods so far is still not sensitive enough to discover very small tumours. In order to solve this limitation, extensive research has been performed to found tumour-related biomarkers in serum sensitive enough to discover small tumours. Thymidine kinase 1 (TK1) is such a tumour-related biomarker. TK1concentration in serum (STK1p) can effectively discover persons with high-risk precancerous diseases that progress to malignant tumours.[1,2] STK1p can also be used to monitor the curative effect, prognosis and risk of recurrence of patients with malignant tumours.[1,2] TK1 in serum discover tumour in the body before discovered by imaging. In patients with breast and colorectal malignancies, it was observed that TK activity in serum was elevated already nine months before manifested by imaging.[3] TK1 is a kinase enzyme that is expressed in the cytoplasm of the cell and converts deoxythymidine (dThd) into deoxythymidine monophosphate (dTMP), a key enzyme introducing dThd into DNA through the salvage pathway, and thus, closely related to cell proliferation.

Although TK1 value has been found to appear earlier than tumour visible by imaging, [1,2] there is still lacking quantitative data on the number of tumour cells needed to release detectable TK1 in serum and also in relation to the size of tumour by imaging. Here we use an automatic chemiluminescence analyser

with high detection accuracy of determining TK1 in serum. This is the first study try to determine the number of cells in the body that releases detectable TK1 into serum.

Study design

In the first part, we investigated whether the automatic chemiluminescence magnetic bead platform is able to determine TK1 in a reliable way. In the second part, we correlate the TK1 concentration in serum (STK1p) to the number of cells that generate detectable concentration of TK1 in serum. In the third part, we show data from health screening in which elevated STK1p correlate to the risk to develop premalignancy and malignancy later in life, before any indication of presence of tumours by imaging. Finally, the results are summary schematic showing the relation between TK1 in serum, imaging of tumours and tumour growth.

Materials And Methods

2.1 Cell growth

$TK1^+$ cell line (human colon tumour, $TK1^+$: ht29) and $TK1^-$ cell line (143B $TK1^-$, Human osteosarcoma cell line, TK1 gene knockout cells) were cultured to logarithmic growth in DMEM (HyClone, China) + 15% foetal bovine serum (Tianhang Bio-Sijiqing Ltd, China) as monolayer. The cells were removed from the plate by 0.05% trypsin. The cell number was counted by Bürker counter camber.

2.2. Cell cycle analysis

TK1 in relation to cell cycle was done in HeLa cells by centrifugation elutriation method as described previously.[4] The proportion of G_1, S-phase and G_2 + M was done by flow cytometric analysis described previously.[4]

2.3. Preparation of cell lysates

After centrifugation of 1ml cell suspension the supernatant was removed and 1ml of cell lysate buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 1% NP40) was added, followed by an incubation at 4°C for 20 min. The supernatant was collected after centrifugation at 15,000 rpm for 10 min. The low TK1 cell lysate ($TK1^-$) was dilute with PBS 10 times, while the high TK1 cell lysate ($TK1^+$) was diluted with PBS 10, 50 and 100 times.

2.4. TK1 standard

A TK1 standard of 31-peptide human TK1 (Hybio Pharmaceutical Co., Ltd. Shenzhen, China) was dissolved in a diluent (10mM Na_2HPO_4, 10mM NaH_2PO_4, 150mM NaCl, 1% BSA, 5% glycerol, pH 7.4) to a concentration of 1mg/ml, adjusting to 0, 1.1, 6.6 and 20 pmol/L (pM) (Fig. 1).

2.5. Serum samples

Serum samples used in this study had no symptoms of tumour-related diseases or no infectious diseases, as assessed by health examination. Some persons might have mild inflammation, or chronic proliferating disease. All samples showed low STK1p values.[5–8] All participants provided informed consent before entering the study, which was conducted in accordance with the Declaration of the 1964 Helsinki Declaration and the Harmonized Tripartite Guideline for Good Clinical Practice from the International Conference on Harmonization. The study shown in Fig. 2 was approved by the ethic committee of Fujun 910 Hospital, Quanzhou, China (No. LL2009003). The results shown in Fig. 5 was performed 2005–2006 in a health screening study in Changsha, China, of a cohort of 11,278 people. At that time ethic permission was not need in China, but permission from local hospitals, which was received.

2.6. Automatic chemiluminescence assay

2.6.1. Preparation of magnetic TK1 particles

The preparation of the magnetic TK1 beads was done according to the company's manual (KeySmart Ltd., China), briefly: 5mg/50µl MS300 Tosyl magnetic beads was add in a magnetic field, removing the supernatant and then adding 2–20 times activation buffer, shaking and washing the magnetic beads. After that, the supernatant was removed and washed again twice for 10 mins.

2.6.2. Couple of antibodies to the magnetic particles

The primary antibody (anti-IgY) was coupled to the magnetic beads by adding at a ratio of 1000:1–10 to a magnetic bead in catalyst and mixing solutions, and then incubated for 18 hours at 37°C. The coupling reaction was blocked by adding 10% BSA and continue to react for six hours at 37°C. The supernatant was removed by put the mixture in a magnetic field, washing the magnetic TK1 beads with a cleaning solution at 4 times, dilute to 1mg/ml, and stored at 2–8°C.

2.7. Antibodies

We used chicken anti-human TK1 IgY polyclonal antibody (HTK1-IgY pAb) raised against a peptide (residue 195–225, GQPAG PDNKE NCPVP GKPGE AVAAR KLFAPQ).[9] Two different batches of the antibody were used for collection and detection of TK1, respectively, in an automatic analyser (Automatic Chemiluminescence Immunoassay Analyser, Keysmile Smart 6500h, China).

2.8. ECL dot blot immune-detection system

A commercial ECL dot blot immune-detection kit was used to determined TK1 in serum (SSTK Inc., Shenzhen, P.R. China, www.sstkbiotech.com). Serum samples were probed with the anti-chicken HTK1-IgY pAb, the same HTK1 IgY pAb as used in the automatic chemiluminescence assay. Briefly, three µl of serum were directly applied onto a nitrocellulose membrane (HybandTM-C, Amersham). TK1 standards (2.2, 6.6 and 20 pmol/L) were used. The membrane was blocked in TBS (Tris-buffered saline) with 6% non-fat milk for 30 mins and incubated at room temperature for 1 h after addition of the primary biotinylated HTK1-IgY pAb. Then the membrane was incubated in TBS buffer with Streptavidin Horse-

Radish-Peroxidase (SA-HRP), followed by addition of Enhanced Chemiluminescence (ECL) substrate. The light intensity of a single spot on the membrane was detected using a CIS-II Imaging System (SSTK Ltd, Shenzhen, China). Based on the light intensities of known concentrations of TK1 standards, the light intensities of the TK1 spots were re-calculated and expressed as pmol/L (pM). All experiments were performed in a blinded manner and in duplicate. [5, 8]

2.9. Western blot

Briefly, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the concentration of TK1 in the various part of the cell cycle of HeLa cells, isolated by centrifugation elutriation.[4]

2.10. TK activity assay

HeLa cells were suspended in cold Tris buffer (100 mM Tris, 70 mM NaCl, 5 mM EDTA, pH 7.5) and washed twice. Cells were then re-suspended to a final concentration of 5×10^6 cells/ml in a lysing buffer (10 mM Tris buffer, pH 7.5, 0.5% P-40 (V/V), 2 mM β-mercaptoethanol, 5 mM NaF, 5 mM $MgCl_{2)}$. The cytoplasmic fraction containing TK1 was obtained by centrifugation at 4,000 *g* for 5 mins and further centrifuged at 48,000 g at 5 mins. Fifty micro-litres of the supernatant were added to 200 µl of a cocktail (55 mM Tris-HCl, pH 7.5, 5.6 mM NaF, 5.0 mM ATP, 3.8 mM $MgCl_2$, and 0.06 mM unlabelled dThd and 2.3 µCi ^{3}H-dThd [specific activity 0.74 TBq or 20 Ci/mmol, New England Nuclear, Boston, MA) and incubated for 60 mins at 37˚C. The mixture was applied to DE-81 paper disks (Whatman) and washed. The radioactivity in the paper disk was measured in a beta-counter. The TK activity was expressed as U/L. Since the TK1 activity is low in G_1, but high in $S + G_2$, the TK activity was also expressed as DPM/$S + G_2$ cells/min. The standard deviation was no more than 10%.[4]

2.11. Statistical analysis

The statistical significance for the correlations between parameters was calculated by correlation-Pearson test (SPSS Statistics V25.0, IBM, USA). P-values of < 0.05 were considered as statistically significant.

Results

3.1. The accuracy of the magnetic bead platform

The accuracy of the chemiluminescence magnetic bead platform was tested by a standard curve (Fig. 1), a recovery test (Table 1), a deviation test (CV) (Table 2) and by distribution of TK1 in serum (Fig. 2).

Table 1
Recovery test experiment.

	Measurement value (pM)	Mean value (pM)	Expected value (pM)	Recovery rate	Acceptable range
Basic sample	2.24	2.25			
	2.24				
	2.23				
Basic sample + 1.10 pM	3.32	3.34	3.35	99.18%	95–105%
	3.37				
	3.31				
Basic sample + 8.00 pM	10.22	10.27	10.25	100.26%	95–105%
	10.31				
	10.28				

Table 2
The deviation test of the chemiluminescence magnetic bead platform.

Quality control sample 1	Quality control sample 2
(2.20 pM)	(10.0 pM)
2.28	10.30
2.27	10.36
2.28	10.29
2.23	10.20
2.22	10.21
2.25	10.19
2.25	10.20
2.26	10.18
2.26	10.21
2.27	10.21
2.28	10.22
2.28	10.21
2.29	10.21
2.29	10.22
2.30	10.19
2.31	10.19
2.30	10.23
2.30	10.19
2.30	10.22
2.28	10.22
CV = 1.01%	CV = 0.44%

3.1.1 Standard curve

In the standard curve, different TK1 concentrations were used corresponding to TK1 values in serum of healthy persons (0-0.5 pM) and malignant patients (1.5–20.0 pM). The correlation coefficient value R^2 was 0.999, showing high accuracy of the magnetic bead platform in the range of TK1 found in serum of normal persons and tumour patients.

3.1.2. Recovery test

The recovery test was performed by determine TK1 concentration before and after adding a known concentration of a 31-TK1 standard peptide (1.10pM and 8.00pM). The TK1 determination was repeated three times. The recovery rates were 99.18% and 100.26%, respectively, within acceptable range (Table 1).

3.1.3. Deviation test

To test the deviation of the automatic magnetic bead device, serum samples containing 2.20 pM or 10.00 pM TK1 were analysed repeatedly 20 times. The deviations were 1.0% and 0.44%, respectively (Table. 2), further showing the accuracy of the chemiluminescence magnetic bead platform.

3.1.4. TK1 distribution in serum

The TK1 concentration in serum of normal healthy people is very low or almost impossible to detect. According to recent health screening data in China, the frequency of disease-free person is only 1–2%. [5–8] Here we collected serum sample from 115 persons who had no symptoms of tumour-related diseases, as assessed by health examination. The TK1 value was almost normal distributed in the range of 0.01-0.60pM, with a tail up to 2.65pM (Fig. 2). The elevated TK1 values in serum above 0.60 is most likely to be of people with mild infections/inflammations or chronic proliferation diseases.[5–8] When compared the chemiluminescence magnetic bead platform assay with the original serum TK1 assay (ECL dot-blot) it was found that the chemiluminescence magnetic bead platform improved the sensitivity three-fold with a sensitivity down to 0.01pM of TK1 concentration in serum. Thus, the chemiluminescence magnetic bead sandwich TK1 kit used in the automatic device is sensitive and specific enough to be able to detect low concentration of TK1 in serum, corresponding to very small tumours.

3.2. TK1 in relation to cell cycle.

To prove that TK1 is a proliferation-biomarker, its relation to cell cycle was performed in HeLa cells. The cell cycle stages were isolated by centrifugated elutriation and determined by flow cytometry DNA measurements, as described previously.[4] TK1 activity and TK1 protein concentration were low in G_1, elevated in S-phase and then decline in G_2 + M (Fig. 3).

3.3. TK1 in growing cells

The TK1 concentration in cells were determined in cell extract of both TK1 low ($TK1^-$) and TK1 high ($TK1^+$) cell lines, growing in culture medium. The TK1 value of the TK1 low-cells contained about 2% of TK1 compared to the TK1 high-cells (Fig. 4). Dilution of the high-TK1 cell extract showed a linear decrease in the OD-values, indicates that the magnetic bead platform is able to measure TK1 of cell extract in a reliable manner. Based on the standard curve, the TK1 concentration per cell was calculated to be 0.021pg. Assuming a total protein concentration per cell of 200pg, a concentration often found in *in vitro* growing cells, TK1 corresponds to 0.01% of total protein concentration of a cell. This is a likely TK1 value and support that the TK1 automatic magnetic platform we use is reliable for measuring TK1 concentration.

3.4. TK1 in relation to cell number and imaging

Recent studies show that TK1 in serum of tumour patients is in the range of 0.1–20pmol/l, (pM).[1,2] In 5 litre blood, a normal blood volume in an adult person, 2pmol/l (pM) of TK1 corresponds to 1.1μg total TK1. Suppose 0.021pg TK1/cell, 1.1μg TK1 in 5 liter blood corresponds to 52.4 x 10^6 growing tumour cells that release TK1 (total 1.1 μg divided by 0.021 pg/cell = 52.4 million cells). That should be compared to the number of cells needed to visible a tumour in imaging of at least 10^9 cells (calculated of a tumour of a diameter of 1mm, containing tumour cells with a diameter of 1μm).

3.5. TK1 in serum (STK1p) correlates to development of malignancy

In the health screening follow-up in this study, we found that elevated STK1p values increase the risk to develop premalignant and malignant tumours and death within 11 years (Fig. 5).

Randomly selected people with low STK1p values (n = 6,352/26,484) and elevated STK1p values (170/702) follow-up 132 months showed a four times higher risk to develop malignancies among people with elevated STK1p values, compared to people with low STK1p values. The risk value of 4 times was calculated from an expected cancer incident rate of 0.2% among the people with low STK1p, based on official cancer statistic in China. If instead use the number of people with new malignant tumours really found in the elevated STK1p group, the risk rate was 47 times. The type of new malignancies appeared in the STK1p elevated group during the 132 months follow-up were gastric, liver, ovarian and prostate carcinomas.

In the elevated STK1p group, 16 persons (9.4%) developed new malignancies (carcinoma of gastric, liver, ovary, prostate) within 132 months and five persons (2.9%) died in their cancer diseases (carcinoma of gastric, liver and breast), within 132 months. Eleven persons (6.5%) showed progress in their pre-malignancy diseases up to 132 months (breast and prostate hyperplasia, HBV of high risk). Twenty three persons did not show any further progress up to 132 months (data not shown). The tumours were discovered by B-ultrasound in the follow-up health screening study and confirmed by imaging.

3.6. Relationships between TK1 and tumour growth in serum

The relationships between TK1 and tumour growth is visualised in Fig. 6, based on data presented here and in previous studies.[1,2] The tumours are visible in imaging when the tumour volume/number of tumour cells excide about 1 mm/10^9 million cells. On the contrary, tumours are detected by TK1 in serum at a concentration of TK1 corresponding to about 50 x 10^6 cells. Hence, TK1 concentration in serum is more sensitive to detect tumours than imaging.

However, it should be noted that these correlations are only found at early and middle stages of tumour growth. In many cases, serum TK1 value decreases in later stages of tumour growth. When the tumour

size increases, and be able to be detected by imaging, the growth rate decreases due to lack of nutrition and as a consequence, reducing the TK1 concentrations in serum.

Discussion

TK1 in serum was determined by an automatic magnetic bead analyser based on chemiluminescence sandwich magnetic bead immune technology, with high sensitivity and accuracy. Chicken anti-human TK1 IgY polyclonal antibodies were used for both catching and detection, produced from different chicken individual, respectively, regarded as different batches of the antibodies. Since we found in preliminary studies that the chicken IgY-IgY antibody sandwich complex showed higher sensitivity and specificity when binding to TK1 in serum compared to mouse TK1 IgY-IgG monoclonal antibody sandwich complex (data not shown), we decided to use IgY antibodies. The TK1 IgY polyclonal antibodies recognise five different epitopes on the C-terminal end of TK1 (1 GQPAGPDNKENCPVP; 2 GEAVAARKLF; 3 NCPVPGKPGEAV; 4 NCPVPGKPGE; 5 GQPAGPDKEN), where three epitopes (No 2, 4, 5) showed significantly higher affinities than the others.[11] This part of TK1 is responsible for the cell cycle regulation of TK1,[12] and thus related to cell proliferation.

The accuracy tests consist of a standard curve, a recovery and a deviation test, showing high accuracy, confirming that the automatic chemiluminescence sandwich magnetic bead immune platform is a reliable method determining TK1 in serum. The deviation was about 1%, which is significantly better than the original serum dot blot TK1 assay (deviation about 15%). The distribution of TK1 concentration in serum also shows that the automatic chemiluminescence sandwich immune magnetic bead platform is sensitive down to 0.01pM. This is crucial, since the TK1 concentration in serum is very low (< 0.10pmol/l (pM)).

Based on the cellular concentration of TK1 (0.021pg/cell), we were able to translate the TK1 concentration in the serum to the number of cells in the body that releases TK1 into the serum. Two pmol/l (pM) TK1 in serum corresponds to about 50 x 10^6 cells in the body that release TK1 into serum, which is about 20 times lower than needed to visualize a tumour in imaging (about 1 mm/10^9 cells). Thus, TK1 in serum is more sensitive than imaging.

In addition, IgY antibodies have the benefit over IgG antibodies because of the interfering with human anti-mouse IgG monoclonal antibody (HAMA),[13] which often causes antibody response effects. In terms of tumour treatment, especially when mouse monoclonal antibodies are used for treatment, the use of monoclonal IgG antibody type, tumour-related markers for detection may increase the incidence of HAMA in the body. The advantage of the chicken antibody detection system to monitor the efficacy of treatment is that it does not react with HAMA. Therefore, immunoassay method using chicken antibodies should theoretically be more reliable than using mammalian antibodies. Furthermore, since there is both genetic and species differences between chicken IgY and human IgG, the risk of unspecific immune reaction is low using chicken IgY antibodies. In addition, IgY antibody does not activate the human complement system, or show Rheumatoid reactions, thereby partially blocking the activation of non-specific antigen

binding sites in human serum. Compared with polyclonal antibodies prepared by traditional rabbit immunization, chicken IgY has endogenous molecular homogeneity (only one type of antibody molecule is produced, namely IgY). Finely, polyclonal antibodies in general show more advantages than single antibody detection because of binding to higher number of epitopes, giving more accurate results.

It is known from recent studies that TK1 is closely related to the cell cycle, and thus related to proliferation.[1,2] The TK1 antibodies we developed and used in this study confirmed that. The level of TK1 starts to increase at the boarder of G_1/S phases of the cell cycle with a maximum value during S-phase and gradually decreases as the cell enters late G_2-phase/mitosis.

TK1 is not a specific tumour-related marker, but a proliferation marker. That means that TK1 is useful both in non-malignant and malignant growing cells. To be able to assess if the elevated TK1 value in serum indicates presence of tumours, we strongly recommended to contact an oncology clinic for further investigation. TK1 in health screening should be regarded as the first warning of the presence of tumours or the risk to develop malignancy later in life. When using TK1 of persons already diagnose for malignancy, TK1 value in serum or in the tumour tissue (immunohistochemistry) indicates the proliferation degree of the tumour and is a reliable value of the prognosis. Our screening study shows that TK1 in serum not only warning for the presence of tumours in the body, but also for the risk to develop malignancies and recurrence and the risk to dye (survival).

Conclusion

TK1 in serum is more sensitive detecting small human tumours compare to imaging. Serum TK1 base on chemiluminescence automatic equipment open up for an effectively assay for screening of early tumour populations, identifying patients with tumour disease risk progression.

Abbreviations

TK1: thymidine kinase1

STK1p: serum thymidine kinase1 protein

dThd: deoxythymidine

dTMP: deoxythymidine monophosphate

IgY: immunoglobulin Y

IgG: immunoglobulin G

BSA: bovine serum albumin

pM: pmol/l

ECL: enhanced chemiluminescence

CV: coefficient of variation

Declarations

Ethics approval and consent to participate

All authors agree to participate in this study. *The clinical study was* approved by the ethic committee of Fujun 910 Hospital, Quanzhou, China (No. LL2009003).

Consent for publication

All authors agree to publish in Cell & Bioscience.

Availability of data and materials

All data are available from the corresponding author.

Competing interests: The authors declare no conflict of interest, expect for JZ who is the president of the company (SSTK Ltd, China) that produce the STK1p kit.

Funding: No funding was available.

Authors' contributions

Ji Zhou: Conception, revision and important intellectual and surveillance of all process and checking the manuscript.

Huijun Li: Preparation of manuscript, mainly for cell culture and cell lysates and checking the manuscript.

Cong Fang, Junye Tan, Peng Gao, Cuicui Jin: Preparation of manuscript, mainly for the automatic chemiluminescence assay and ECL do blot assay and checking the manuscript.

Sonbo Liu: Preparation of manuscript, mainly for collection of serum samples and ECL do blot assay and checking the manuscript.

Yu Wang: Preparation of manuscript, mainly for collection of serum samples and checking the manuscript.

Jin Li: Surveillance of all process and checking the manuscript.

Ellen He: Conception, design of the study and preparation of manuscript, including TK1 western blot analysis and TK activity assay, analysis of all results and checking the manuscript.

Sven Skog: Conception, design of the study and preparation of manuscript, mainly for analysis of cell cycle, inspection of all results and writing the manuscript.

Conflict of interest

No conflict of interest was reported, except for JZ, who is the president of Sino-Swed Tongkang Bio-Tech, Inc., Shenzhen, China, that produce the TK1 biomarker.

Acknowledgements

We thank the support from the Shenzhen Ellen-Sven Precision Medicine Institute, Shenzhen, China; the Karolinska University Hospital and the Karolinska Institute, Sweden. We also thank Li Dang and Lewen Liang for technical assistant running the automatic magnetic equipment.

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Figures

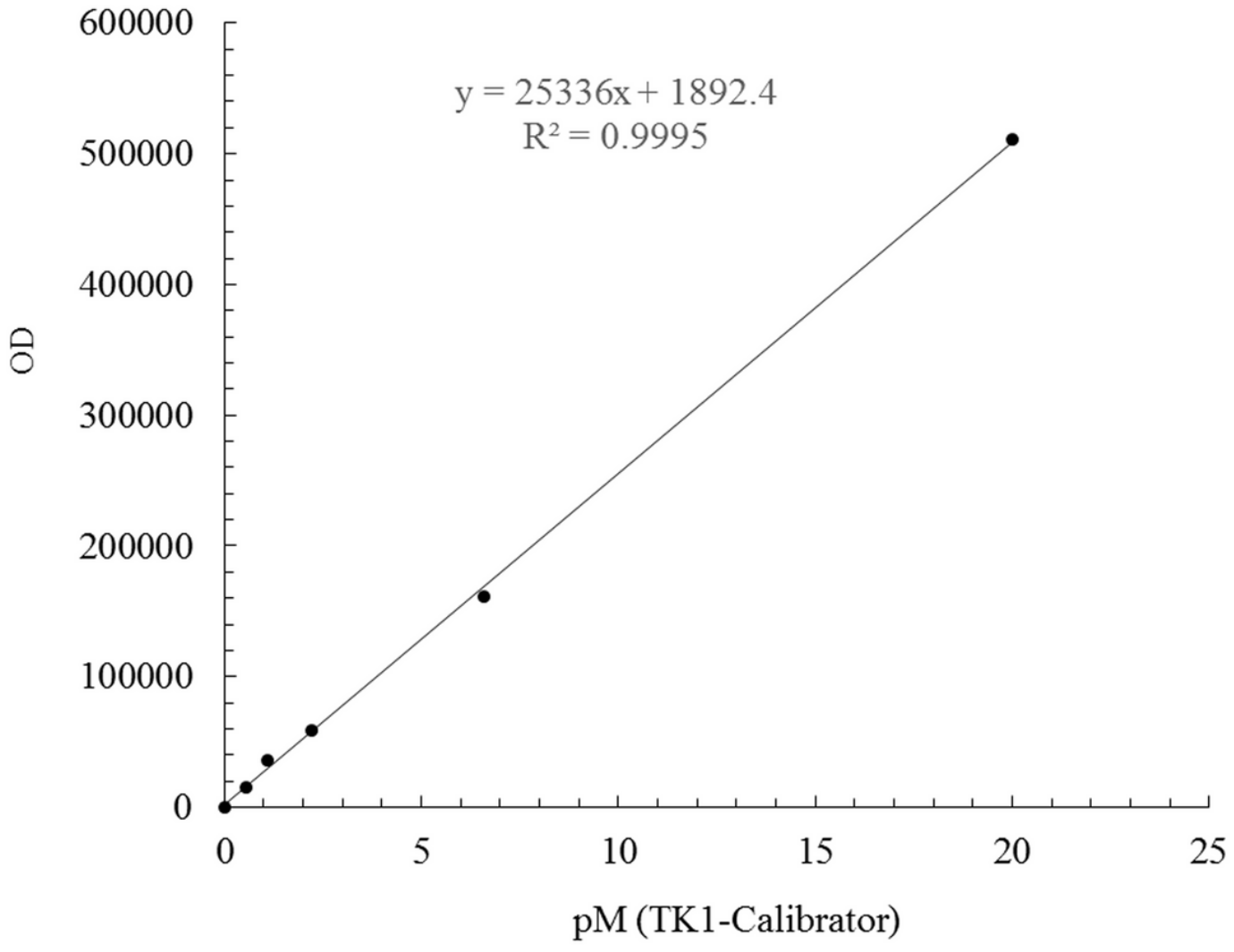

Figure 1

TK1 standard curve.

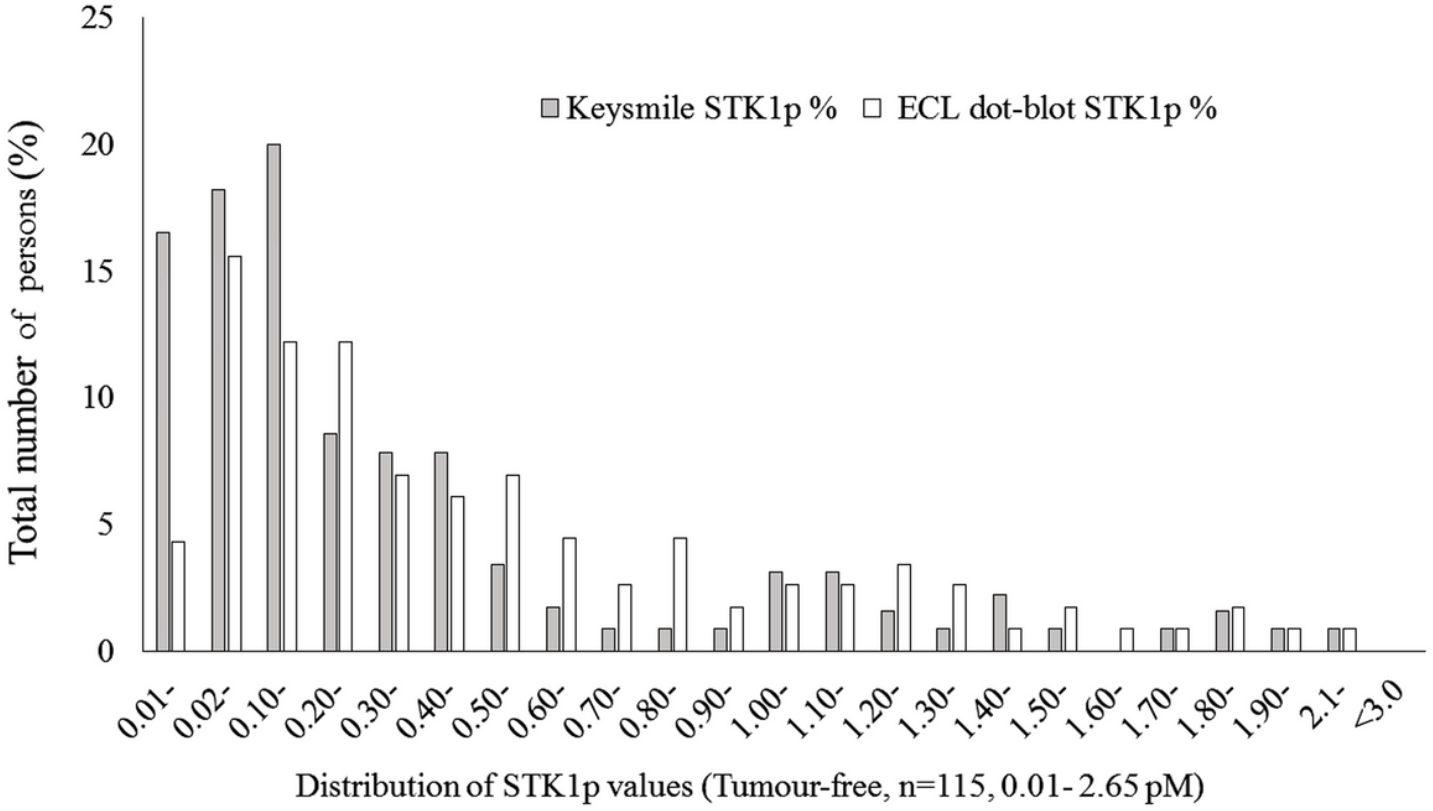

Figure 2

STK1p distribution determined by a chemiluminescence magnetic bead platform assay (Keysmile) and a ECL dot blot immune-detection system. The serum samples (n=115) were collected in a health screening study in Fujin, China, of a cohort of 160,000 people.

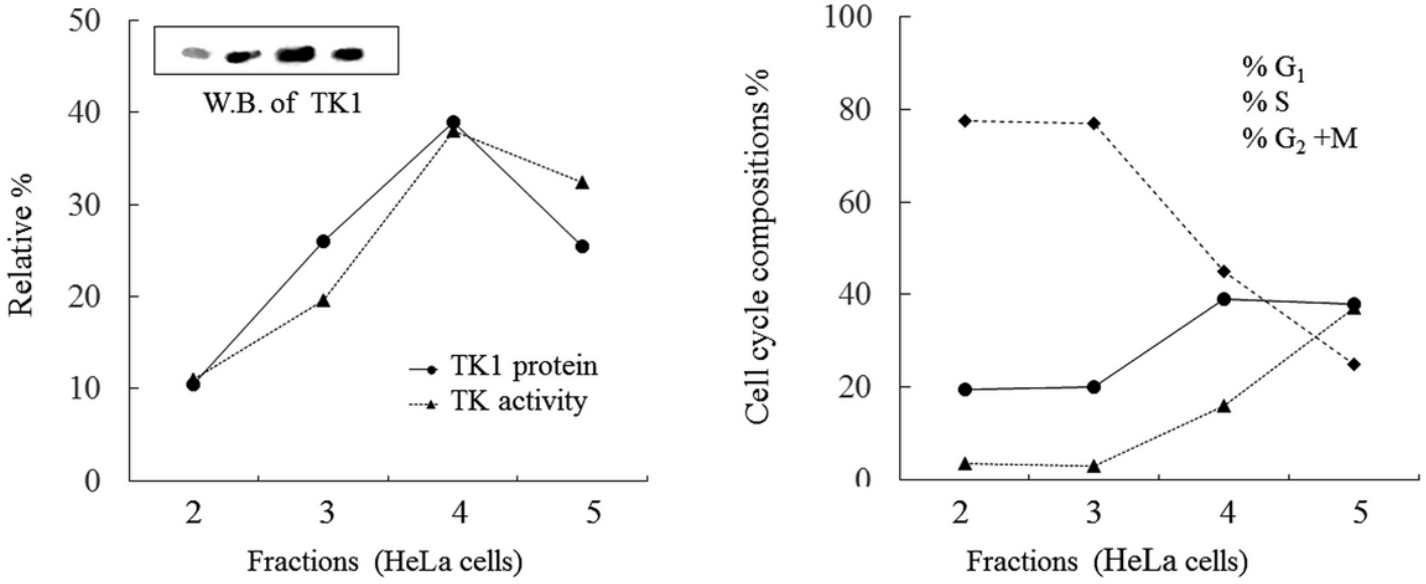

Figure 3

TK1 in relation to cell cycle. W.B.:Western Blot.

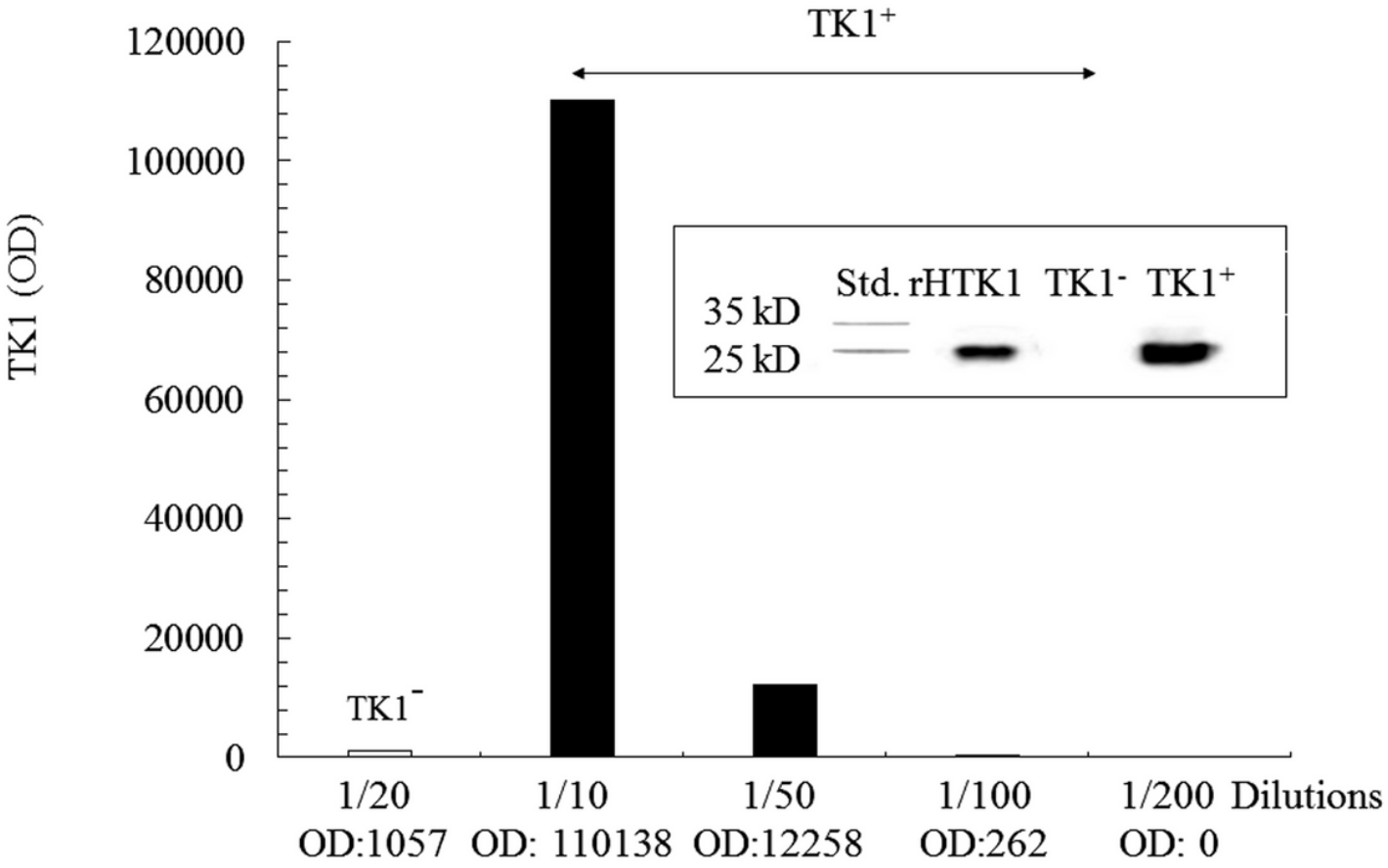

Figure 4

Concentration of TK1- and TK1+ cell lines and Western Blot.

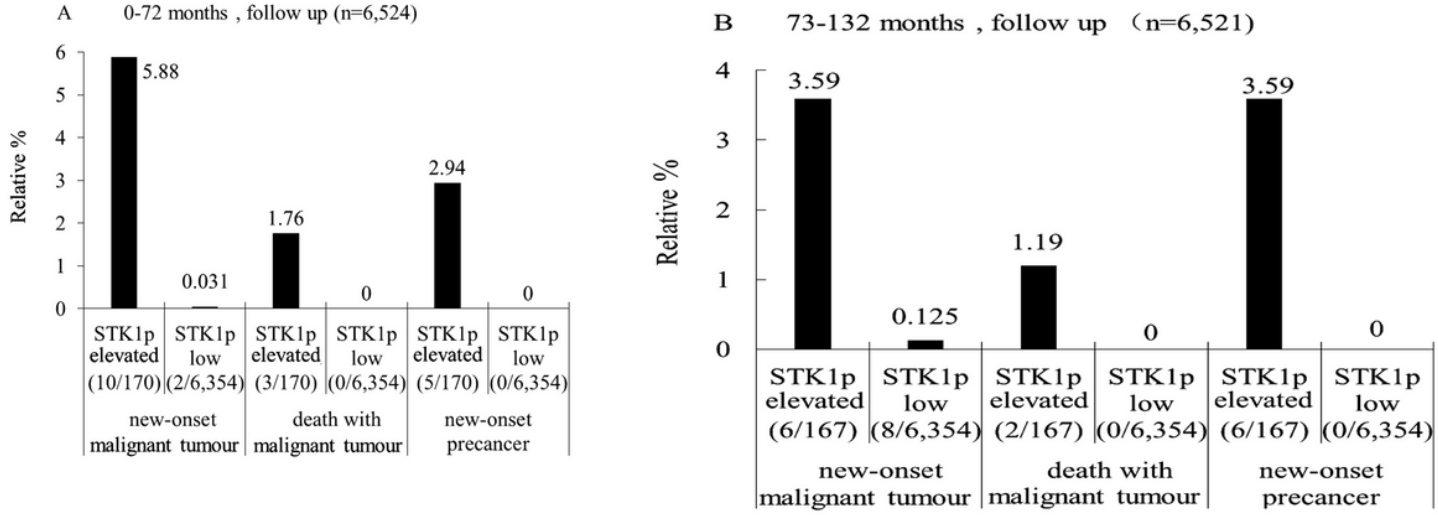

Figure 5

Elevated STK1p in relation to risk to develop pre- and malignant tumours and death. A) Follow-up 0-72 months, B) follow-up 73-136 months. Data published with the permission from E. He and S. Haghdoost,

the copywrite holders, (see reference 1). The serum samples were collected in a health screening study at the Health Management Centre, Central South University, Changsha, China, of a cohort of 11, 278 people.

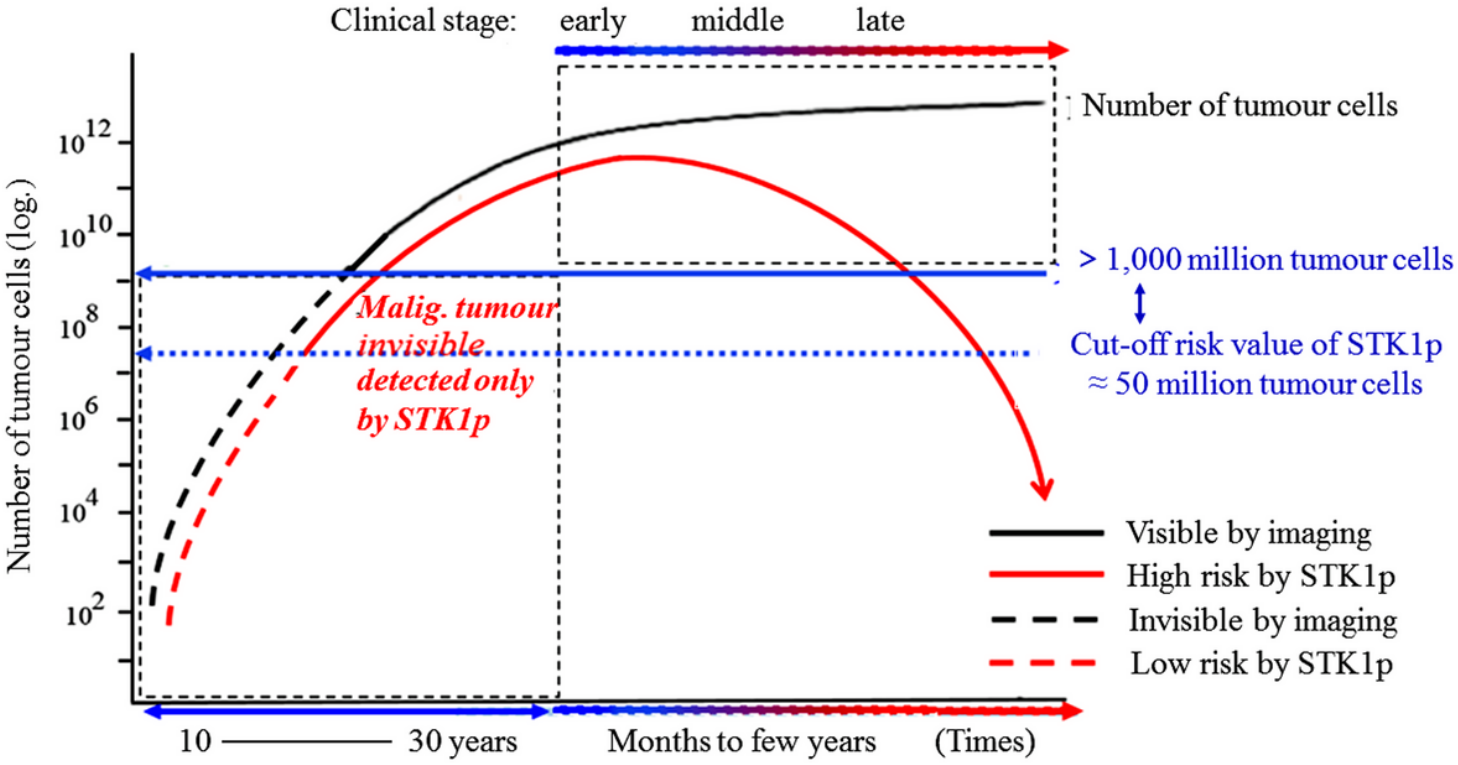

Figure 6

A schematic diagram of correlation between human TK1 concentration in serum and tumour growth. Clinical stages refer to visible tumours by imaging.